Chapter 8
TGF-β Signaling in Leukemogenesis

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Abstract Transforming growth factor-β (TGF-β) signaling plays diverse roles in regulating cell proliferation, differentiation, and "stemness" in a cell context-dependent manner. In the first section of this chapter, we outline the genetic changes that attenuate TGF-β signaling in patients with acute myelogenous leukemia (AML), or the blast crisis phase of chronic myelogenous leukemia (CML), or pediatric acute lymphoblastic leukemia (ALL). In the second section, we discuss recent advances in stem cell research indicating that TGF-β signaling does not always suppress leukemogenesis. In fact, TGF-β signaling paradoxically sustains the survival and resistance to therapy of the CML stem cells that are responsible for disease recurrence in CML patients treated with tyrosine kinase inhibitors (TKIs). We examine evidence implicating TGF-β and its downstream effectors Akt and FOXO in the in vivo maintenance of the self-renewal ability of both TKI-resistant CML stem cells and the normal hematopoietic stem cells (HSCs) from which they are derived. Increased knowledge of the complex effects of TGF-β signaling may lead to improved diagnostic and therapeutic tools that can benefit leukemia patients.

Keywords TGF-β • Smad • FOXO • CML stem cells • TKI-resistance

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Abbreviations

ALL	Acute lymphoblastic leukemia
AML	Acute myelogenous leukemia
ANLL	Acute nonlymphoblastic leukemia
APL	Acute promyelocytic leukemia
ATRA	All-*trans* retinoic acid
BMP	Bone morphogenetic protein
Bmpr1a	BMP receptor type 1A
CCyR	Complete cytogenetic response
CML	Chronic myelogenous leukemia
cPML	Cytoplasmic isoform of PML
FAB	French–American–British
GFAP	Glial fibrillary acidic protein
HSC	Hematopoietic stem cell
KIR	Killer immunoglobulin-like receptor
L-GMP	Leukemic-granulocyte-macrophage progenitor
LRC	Lipid raft clustering
LSK	$Lineage^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$
MEF	Mouse embryonic fibroblast
MPN	Myeloproliferative neoplasm
NK	Natural killer
PI3K	Phosphatidylinositol-3-OH kinase
PML	Promyelocytic leukemia
RARα	Retinoic acid receptor α
ROS	Reactive oxygen species
SARA	Smad anchor for receptor activation
SCL	Stem cell leukemia
SOD	Superoxide dismutase
TGF-β	Transforming growth factor-β
TKI	Tyrosine kinase inhibitor
WHO	World Health Organization

8.1 Genetic Alterations Affecting the TGF-β-Smad Pathway in Leukemia Patients

The transforming growth factor-β (TGF-β) family of highly conserved and highly homologous secreted polypeptides (Massagué et al. 1990) was described in Chap. 1. Briefly, TGF-β molecules bind to two heteromeric cell surface receptors with serine/threonine kinase activity: TGF-β receptor type I (TGF-βRI) (also known as activin receptor-like kinase 5; ALK5) and TGF-β receptor type II (TGF-βRII) (Massagué 2000). Binding of TGF-β to TGF-βRII triggers the recruitment and

activation of TGF-βRI, which phosphorylates downstream Smad proteins (Heldin et al. 1997). That TGF-β signaling is involved in leukemogenesis has been known (Blank and Karlsson 2011; Dong and Blobe 2006; Kim and Letterio 2003; Larsson and Karlsson 2005). It has been known that TGF-β signaling pathway is altered in hematological malignancies such as acute myelogenous leukemia (AML) and final fatal phase of chronic myelogenous leukemia (CML) termed "blast crisis." AML is a heterogeneous clonal disease characterized by rapid accumulation of abnormal immature white blood cells in the bone marrow and peripheral blood. Although chronic phase CML is a relatively benign myeloproliferative neoplasm (MPN), CML patients in blast crisis reveal rapid proliferation of immature leukemic blasts which are resistant to therapy. In addition, genetic changes that attenuate TGF-β signaling also drive the excessive cell proliferation that characterizes acute lymphoblastic leukemia (ALL) originated from T-cell and B-cell lineage.

In this section of this chapter, we examine this assumption in the context of major chromosomal alterations that affect TGF-β signaling in human leukemia patients.

8.1.1 Acute Myelogenous Leukemia

8.1.1.1 t(8;21)(q22;q22)-Positive AML

The traditional French–American–British (FAB) classification system used to diagnose AML patients in twentieth century was based on cellular morphology and cytochemistry. Starting in 2001, a new World Health Organization (WHO) classification system that takes into account cell morphology, patient and clinical factors, and genetic and chromosomal alterations has been used to provide a scientific basis for AML diagnosis (Jaffe et al. 2001; Swerdlow et al. 2008). The chromosomal translocation t(8;21)(q21.3;q22.12), which generates the AML1–ETO fusion oncoprotein, is a causal genetic change in cases of "immature" type AML (defined as M0 in the FAB system). The normal AML1 protein, also known as Runx1, CBFA2 or PEBP2αB, is a member of the *runt* family of transcription factors. In mammalian embryos, AML1 is critical for the development of definitive hematopoiesis. In adults, AML1 is dispensable for the maintenance of normal hematopoietic stem cells (HSCs) but required for the maturation of megakaryocytes and the differentiation of T- and B-lymphoblasts (Ichikawa et al. 2004). The normal ETO protein, also known as MTG8, is involved in transcriptional regulation because it recruits corepressors such as N-CoR, mSin3A, and SMRT. In the AML1–ETO fusion oncoprotein, a critical lysine residue is acetylated by the transcriptional coactivator p300, an event implicated in AML initiation (Wang et al. 2011). Importantly, AML1–ETO interacts with Smad3 and inhibits its ability to bind to DNA, suppressing TGF-β-Smad signaling (Jakubowiak et al. 2000) (Fig. 8.1). These results support the hypothesis that AML1–ETO drives AML leukemogenesis because it abrogates TGF-β-mediated tumor-suppressive signaling.

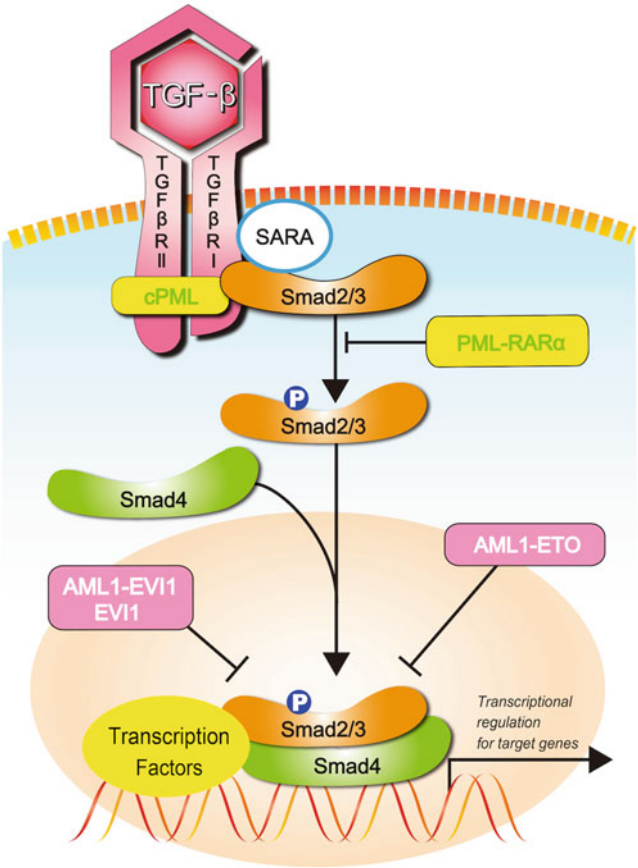

Fig. 8.1 The role of TGF-β signaling in human leukemogenesis. TGF-β signaling is transduced by a cytoplasmic complex containing TGF-β type I receptor (TGF-βRI), TGF-β type II receptor (TGF-βRII), Smad2/3, SARA and cPML. Binding of TGF-β to TGF-βRII triggers the activation of TGF-βRI, which phosphorylates downstream Smad2/3 proteins. The phosphorylated Smad2/3 molecules interact with Smad4, which regulates the expression of genes able to suppress leukemogenesis in the nucleus. The PML–RARα fusion oncoprotein, which causes APL, inhibits cPML function and blocks tumor-suppressive TGF-β signaling. Similarly, the AML1–ETO oncoprotein expressed in AML patients and the AML1–EVI1 oncoprotein or normal EVI1 protein expressed in blast crisis CML patients interact with Smad3 proteins, interfering with their DNA-binding capacity and abrogating TGF-β-mediated growth arrest

8.1.1.2 t(15;17)(q22;q21)-Positive Acute Promyelocytic Leukemia

Acute promyelocytic leukemia (APL, defined as M3 in the FAB classification) is a subtype of AML characterized by the accumulation of abnormal granulated promyelocytes in the bone marrow and peripheral blood. The causal t(15;17)(q24.1;q21.2) translocation generates an oncoprotein (PML–RARα) in which the promyelocytic leukemia (PML) protein is fused to the retinoic acid receptor α (RARα). The normal

PML protein, which is essential for the formation of a dot-like nuclear structure called the "PML nuclear body," has been implicated in transcription, maintenance of genomic integrity, antiviral responses, apoptosis induction, tumor suppression, and stemness. The normal RARα protein belongs to the nuclear receptor superfamily and transduces all-*trans* retinoic acid (ATRA)-mediated transcriptional signaling. APL patients treated with ATRA show PML–RARα degradation and enhanced differentiation of APL cells. Mouse embryonic fibroblasts (MEFs) obtained from *Pml*-deficient mice are resistant to TGF-β-dependent growth arrest, suggesting that PML is involved in the activation of the TGF-β pathway (Lin et al. 2004). Indeed, Lin et al. (2004) reported that a cytoplasmic isoform of PML (cPML) is crucial for TGF-β signaling. The cPML protein participates in the complex containing TGF-βRI, TGF-βRII, Smad2/3, and the "Smad anchor for receptor activation" (SARA) proteins, thereby promoting TGF-β signal transduction. The expression of PML–RARα inhibits cPML function and blocks TGF-β signaling (Fig. 8.1). In *Pml*-deficient MEFs, restoration of cPML function restores normal TGF-β-dependent growth arrest, cellular senescence, and apoptosis. Treatment of APL cell line, NM4, with ATRA increased the interaction between cPML and Smad2/3 and improved their TGF-β responses, implicating PML–RARα-mediated inhibition of TGF-β signaling in APL leukemogenesis (Lin et al. 2004).

8.1.2 Blast Crisis CML

Human CML is a myeloproliferative neoplasm (MPN) that occurs in three phases: (1) an initial chronic phase, (2) an accelerated phase, and (3) finally a blast crisis phase (Ren 2005). The majority of CML patients suffer from the t(9;22)(q34;q11.2) translocation that generates the Philadelphia chromosome and the *BCR–ABL* fusion oncogene, which encodes a constitutively active tyrosine kinase (de Klein et al. 1982; Rowley 1973). Because chronic phase CML cells can still produce functionally normal mature blood cells similar to those in healthy individuals (Fialkow et al. 1977), newly diagnosed CML patients exhibit a massive expansion of myeloid cells that extends for 5–15 years (chronic phase). The acquisition of additional genetic changes and/or epigenetic alterations during this period drives the progression of the disease to the accelerated phase. In the last and lethal blast crisis phase, cells of the myeloid, B-lymphoid, or bi-phenotypic myeloid, and lymphoid mixed lineage are observed (Calabretta and Perrotti 2004).

Blast crisis CML is associated with expression of the AML1–EVI1 fusion oncoprotein generated by the t(3;21)(q26.2;q22.12) translocation. The normal EVI1 protein encodes a zinc-finger transcription factor known to regulate HSC maintenance (Goyama et al. 2008; Yuasa et al. 2005). Excessively high levels of the normal EVI1 protein alone can also cause blast crisis CML (Mitani et al. 1994; Ogawa et al. 1996). Notably, both the normal EVI1 protein and the AML1–EVI1 fusion oncoprotein interact with Smad3 (Fig. 8.1). This association attenuates the DNA-binding activity of Smad3 and thus inhibits the TGF-β signaling pathway (Kurokawa et al. 1998a, b). These findings indicate that loss of TGF-β signaling is implicated in the

progression of CML from the chronic phase to blast crisis and that this interference with TGF-β signaling is due to the interaction of Smad3 with EVI1 or AML1–EVI1.

8.1.3 T-Cell ALL

Letterio and colleagues have examined Smad2/3 proteins in a spectrum of patients with various forms of childhood ALL including T-cell ALL, pre-B-cell ALL, and acute nonlymphoblastic leukemia (ANLL) (Wolfraim et al. 2004). Although Smad2 protein is present at normal levels in leukemic cells of all ALL subtypes examined, Smad3 protein is low in leukemic cells from T-cell ALL patients but not in leukemic cells from pre-B cell ALL or ANLL (Wolfraim et al. 2004). However, Smad3 mRNA has been present at normal levels in leukemic cells from all T-cell ALL patients. Furthermore, no evidence for a deletion or point mutation in any region of the all nine exon of the Smad3 gene (*MADH3*) has been found in any T-cell ALL patients. Nevertheless, although the molecular mechanism responsible for the deficiency of Smad3 protein in these patients has yet to be elucidated, it is tempting to speculate that it might underlie the leukemogenesis of pediatric T-cell ALL. In mice, the loss of a single *Smad3* allele impairs the growth-suppressive effects of TGF-β on normal T-cells (Wolfraim et al. 2004). Moreover, mice harboring a heterozygous deletion of *Smad3* plus homozygous loss of $p27^{Kip1}$ spontaneously develop T-cell leukemia (Wolfraim et al. 2004). These results suggest that a combination of attenuated TGF-β signaling due to *Smad3* deficiency and the homozygous inactivation of $p27^{Kip1}$ might cause pediatric ALL. Indeed, deletions and germline mutations of the *CDKN1B* gene encoding the $p27^{Kip1}$ protein are frequent in pediatric ALL patients (Komuro et al. 1999; Takeuchi et al. 2002).

8.1.4 B-Cell ALL

The t(12;21)(p13.2;q22.12) translocation generating the fusion oncoprotein TEL–AML1 (also known as ETV6–RUNX1) is found in patients with childhood B-cell precursor ALL. Like AML1–EVI1 in AML patients, TEL–AML1 binds to Smad3 and compromises its ability to activate transcription of its target genes (Ford et al. 2009). Murine B-cell progenitor cells expressing TEL–AML1 proliferate more slowly than parental cells but are more resistant to TGF-β-mediated growth suppression (Ford et al. 2009). Importantly, mice expressing a *TEL–AML1* transgene show a greatly expanded number of pre-pro-B cells (Ford et al. 2009). Thus, whereas expression of TEL–AML1 alone is insufficient for the development of full-blown leukemia, this oncoprotein can promote the proliferation of preleukemic cells.

Section summary: The available evidence supports a tumor-suppressive function for TGF-β signaling, at least in differentiated leukemia cells.

8.2 Role of TGF-β-FOXO Signaling in CML Stem Cells

Although TGF-β mediates cytostatic signaling in normal and/or premalignant epithelial cells, it has been clarified that TGF-β promotes their own proliferation, invasion, and metastatic behavior in advanced malignant cells in epithelial-derived tumors (Ikushima and Miyazono 2010; Massagué 2008, 2012; Wakefield and Roberts 2002). In contrast to the case of solid tumor, it has been supposed that suppression of TGF-β signaling pathway is a cause of hematopoietic malignancy, including AML, ALL, and blast crisis CML as described in Sect. 8.1. However, TGF-β signaling and its triggering of the downstream Akt–FOXO pathway paradoxically regulates the self-renewal of CML stem cells and has been linked with their therapeutic resistance. In CML patients with recurrent disease, the reappearance of leukemia is due to the generation of a large number of differentiated CML cells from a rare population of therapy-resistant CML stem cells. Because CML stem cells originate from normal HSCs, it has been suggested that HSCs and CML stem cells share the same molecular mechanisms for regulating the maintenance of their self-renewal ability. In this section of this chapter, we examine the roles of TGF-β and Akt–FOXO signaling in CML stem cells and normal HSCs.

8.2.1 Shared Hallmarks of HSCs and CML Stem Cells

Several lines of evidence implicate normal HSCs as the cell of origin for CML stem cells expressing *BCR–ABL*. (1) Chronic phase CML cells can give rise to multiple lineages of functionally normal mature blood cells (Fialkow et al. 1977), indicating that these CML cells retain normal pluripotent differentiation capacity. (2) The transplantation of murine HSCs engineered to express the human *BCR–ABL* gene induces CML-like MPN in recipient mice (Daley et al. 1990; Elefanty and Cory 1992; Gishizky et al. 1993; Kelliher et al. 1990; Pear et al. 1998). In contrast, the transplantation of murine committed hematopoietic progenitor cells expressing *BCR–ABL* does not induce CML (Huntly et al. 2004). (3) CML stem cells are functionally defined by their ability to induce CML once transplanted into recipient mice. In mice that develop CML-like disease due to retroviral transduction of the *BCR–ABL* gene, CML stem cells can be purified from a rare Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) population (i.e., cells that bear the marker profile of normal HSCs) (Hu et al. 2006; Ito et al. 2008; Zhao et al. 2007, 2009). (4) In an inducible *BCR–ABL* transgenic mouse model in which expression of the *BCR–ABL* gene is controlled by a Tet-regulated 3′-enhancer of the murine *SCL* (stem cell leukemia) gene (Huettner et al. 2003; Koschmieder et al. 2005), the CML stem cells driving disease are restricted to populations with the phenotype of long-term HSCs (i.e., CD34$^-$Flt3$^-$LSK or CD150$^+$Flt3$^-$CD48$^-$LSK) (Reynaud et al. 2011; Schemionek et al. 2010; Zhang 2012). (5) In chronic phase CML patients, the CML stem cells responsible for the disease can be isolated from a cell population expressing surface markers associated

with primitive hematopoietic cells (Holyoake et al. 1999, 2001; Pellicano et al. 2009). These observations collectively bolster the hypothesis that HSCs and CML stem cells regulate their self-renewal ability using the same mechanisms. Indeed, several signaling pathways, including those involving Wnt-β-catenin (Scheller et al. 2006; Zhao et al. 2007), PML (Ito et al. 2008), and Hedgehog (Dierks et al. 2008; Zhao et al. 2009), have been implicated in the regulation of stem cell fate during both normal hematopoiesis and CML leukemogenesis. Some researchers have taken these similarities in properties between HSCs and CML stem cells as evidence that CML is a "stem cell disease" (Naka et al. 2010a) and therefore contend that elucidating mechanisms in HSCs will lead to a better understanding of how CML stem cells are perpetuated.

8.2.2 Akt–FOXO Signaling in HSCs

HSCs top the hierarchy of the normal hematopoietic system and are defined by both their ability to reproduce themselves, a property known as self-renewal, and their capacity to give rise to all mature hematopoietic cell lineages. Because HSCs have to sustain hematopoiesis for the life of an individual, the maintenance of HSC quiescence in vivo is crucial for homeostasis. HSC quiescence is adopted within defined "bone marrow niches," which provide a comfortable microenvironment where HSCs can remain in the non-dividing, G0 phase of the cell cycle (Suda et al. 2005). Quiescent HSCs generate only very low levels of reactive oxygen species (ROS), allowing these cells to escape any DNA damage that might be caused by oxidative stress. Genomic integrity is thought to be crucial for the preservation of HSC self-renewal capacity, and it is speculated that dysfunction of genomic integrity causes generation of the Philadelphia chromosome and thereby *BCR–ABL* fusion oncogene.

The self-renewal of normal HSCs also depends on signaling involving the phosphatidylinositol-3-OH kinase (PI3K)–Akt pathway and its downstream targets FOXO1, FOXO3a, FOXO4, and FOXO6, which are members of the Forkhead O (FOXO) subfamily of transcription factors (Greer and Brunet 2005). In the absence of stimulation by growth factors or insulin, FOXOs are present in an active state in a cell's nucleus and freely induce their transcriptional targets. When a growth factor or insulin binds to the appropriate cell surface receptor, PI3K–Akt signaling is activated, and activated Akt directly phosphorylates FOXOs, resulting in their nuclear exclusion and subsequent degradation in the cytoplasm.

FOXO deficiencies have profound effects on HSCs. For example, Tothova et al. reported a marked decrease in HSCs in mice with triple conditional deletions of the *Foxo1*, *Foxo3a*, and *Foxo4* genes (Tothova et al. 2007). The HSCs from these triple *Foxo*-deficient mice not only exhibited a self-renewal defect but also showed elevated ROS. Notably, the expression of superoxide dismutase (*SOD*) genes was impaired in triple *Foxo*-deficient HSCs. In two other studies, Foxo3a was found to be solely responsible for the maintenance of the HSC pool (Miyamoto et al. 2007;

Yalcin et al. 2008). *Foxo3a*$^{-/-}$ HSCs showed reduced expression of the Foxo target genes *SOD2* and *catalase* and exhibited increased ROS, defective self-renewal, and decreased expression of the CDK inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ (Miyamoto et al. 2007; Yalcin et al. 2008). Importantly, $p57^{Kip2}$ and $p27^{Kip1}$ are required for the maintenance and quiescence of HSCs (Matsumoto et al. 2011; Zou et al. 2011). Thus, the FOXO factors are responsible for regulating HSC quiescence, ROS levels and maintenance of self-renewal ability.

The Akt–FOXO pathway in quiescent HSCs within a bone marrow niche is regulated by lipid raft clustering (LRC) (Yamazaki et al. 2009). Lipid raft microdomains, which are patches in the plasma membrane enriched in cholesterol, glycosphingolipids, and glycolipids, control cytokine signaling in murine HSCs. Yamazaki et al. demonstrated that HSCs freshly isolated from mouse bone marrow lacked LRC. Notably, LRC-induced cytokine signaling is essential for the cell cycle reentry of HSCs. Conversely, inhibition of LRC attenuates cytokine signaling, which results in repression of Akt activity followed by Foxo activation. Therefore, the status of the lipid rafts within HSCs in a bone marrow niche fine-tunes Akt–Foxo signaling and regulates HSC quiescence.

8.2.3 TGF-β Signaling in HSCs

Numerous in vitro studies have indicated that TGF-β signaling is involved in regulating mouse and human HSCs (Larsson and Karlsson 2005). Yamazaki et al. clarified that TGF-β signaling controls HSC self-renewal ability by inhibiting LRC and thereby attenuating cytokine signaling (Yamazaki et al. 2009). The inhibition of LRC by TGF-β in HSCs suppressed Akt and induced nuclear localization of Foxo3a. These results link TGF-β signaling with the LRC–PI3K–Akt–Foxo signaling pathway that regulates HSC quiescence in vivo.

While it had been hoped that a genetic approach might reveal the precise role of TGF-β in modulating HSCs in vivo, it has been difficult to carry out the required experiments due to the early lethality and massive inflammatory response in null mutant mice disrupted in the *TGF-β1* (Kulkarni et al. 1993; Shull et al. 1992), *TGF-βRI* (Larsson et al. 2001), or *TGF-βRII* (Oshima et al. 1996) genes. To overcome these difficulties, conditional knockout mice with hematopoietic cell-specific loss of the *TGF-βRI* (*ALK5*) or *TGF-βRII* genes were established using the Cre/LoxP system. Interestingly, while conditional mutants lacking *TGF-βRI* (*ALK5*) had no significant defects in the quiescence and maintenance of HSC pool (Larsson et al. 2003, 2005), *TGF-βRII*-deficient conditional mutants exhibited obvious abnormalities in HSCs (Yamazaki et al. 2011). Similarly, HSCs of conditional *Smad4*-deficient mice showed a significant reduction in self-renewal capacity following transplantation into normal recipient mice (Karlsson et al. 2007). Interestingly, although Smad4 plays an integral role in both the TGF-β and bone morphogenetic protein (BMP) pathways, studies of conditional mutant mice disrupted in the *Smad1* and *Smad5* genes revealed that canonical BMP signaling is

dispensable for HSC maintenance (Singbrant et al. 2010). Thus, it is the TGF-β-Smad pathway that is required for HSC maintenance in vivo (Yamazaki et al. 2011). However, conditional disruption of the *BMP receptor type 1A* (*Bmpr1a*) gene in mice results in an increased number of osteoblasts, which expand the size of the HSC bone marrow niches. This expansion in turn facilitates the enlargement of the HSC pool, indicating that BMP signaling is indirectly involved in the control of HSC numbers (Zhang et al. 2003).

HSCs themselves produce TGF-β in a latent form but are not able to process it into its active structure. Yamazaki et al. have shown that bone marrow glial cells expressing glial fibrillary acidic protein (GFAP) regulate the maintenance of quiescent HSCs by activating their latent TGF-β (Yamazaki et al. 2011). These GFAP-expressing glial cells have been identified as non-myelinating Schwann cells that ensheath sympathetic nerves in the bone marrow. Importantly, when the number of active TGF-β-producing cells is reduced via autonomic nerve denervation, HSCs are missing from the bone marrow (Yamazaki et al. 2011). Taken together, these results indicate that the signaling of TGF-β family members within the bone marrow niche has profound regulatory effects on HSC properties.

8.2.4 Opposing Roles of FOXOs in CML Non-stem Cells vs. CML Stem Cells

Given that CML stem cells originate from normal HSCs, it has been of great interest to determine whether CML stem cells conserve the regulatory mechanisms found in normal HSCs. Studies using CML cell lines have shown that, in CML non-stem cells, activated FOXOs can induce apoptosis or cell cycle arrest (Essafi et al. 2005; Ghaffari et al. 2003; Komatsu et al. 2003). The BCR–ABL fusion protein in CML cells drives strong Akt activation that leads to nuclear export of Foxo3a and forceful repression of Foxo3a functions (Essafi et al. 2005; Ghaffari et al. 2003; Komatsu et al. 2003). When TKIs block BCR–ABL and suppress Akt activity, Foxo3a remains activated and triggers apoptosis or cell cycle arrest (Fig. 8.2, left). Based on these findings, it has been believed that FOXOs are suppressors of CML leukemogenesis. However, Komatsu et al. insightfully reported several years ago that Foxo3a is involved in the acquisition of dormancy in CML cell lines exposed to imatinib (Komatsu et al. 2003). Indeed, when we investigated whether Foxo3a was similarly implicated in the maintenance of CML stem cells using a mouse CML model, we found that, unlike CML non-stem cells, CML stem cells exhibit decreased Akt phosphorylation and increased nuclear localization of Foxo3a in vivo (Naka et al. 2010b). In line with this observation, Lee et al. reported that Foxo3a was localized in the nucleus of a primitive $CD150^{+}CD48^{-}CD41^{-}$LSK population in a *Pten-deficient* MPN mouse model (Lee et al. 2010). Thus, it appears that the maintenance of the rare CML stem cell population is controlled by different regulators than those governing the majority of CML non-stem cell population.

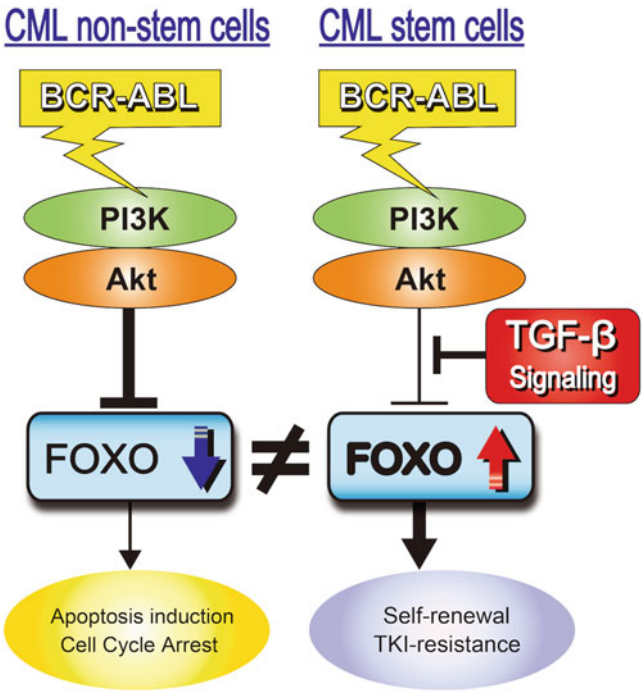

Fig. 8.2 Opposing roles of the TGF-β-FOXO axis in CML stem cells vs. CML non-stem cells. FOXO transcription factor is generally thought to suppress tumorigenesis because activated FOXO induces the apoptosis or cell cycle arrest. In CML non-stem cells (*left*), FOXO functions are suppressed by BCR–ABL, which drives the activation of PI3K–Akt signaling pathway. In CML non-stem cells treated with TKIs, BCR–ABL activity is blocked, Akt activity is suppressed, and FOXOs remain activated. Differentiated leukemic cells are thus reduced in number. Paradoxically, in CML stem cells (*right*), the TGF-β-FOXO axis is tumor-promoting. BCR–ABL-triggered activation of the PI3K–Akt signaling pathway is inhibited by TGF-β signaling. As a consequence, FOXOs remain active and drive the self-renewal and TKI resistance of CML stem cells. Because BCR–ABL does not function in CML stem cells, TKIs have no effect on them and cannot eradicate them. Thus, it is possible that the therapeutic suppression of the TGF-β-FOXO axis might be a novel strategy for the eradication of CML stem cells and thus the inhibition of leukemia recurrence that is frequently lethal

To clarify the functional role of Foxo3a in CML stem cells, we transplanted *Foxo3a*$^{+/+}$ and *Foxo3a*$^{-/-}$ CML stem cells into recipient mice (Naka et al. 2010b). Both groups of recipients died of CML-like MPN with similar kinetics, indicating that Foxo3a is dispensable for the initiation of CML. However, serial transplantation of *Foxo3a*$^{+/+}$ and *Foxo3a*$^{-/-}$ CML stem cells revealed that the ability of CML stem cells to perpetuate disease in vivo is significantly decreased by *Foxo3a* deficiency. Moreover, recipients that received *Foxo3a*$^{+/+}$ CML stem cells developed both ALL and CML, demonstrating that these CML stem cells are able to generate malignancies in multiple hematopoietic lineages. In contrast, recipients transplanted with *Foxo3a*$^{-/-}$ CML stem cells developed neither ALL nor CML. Thus, Foxo3a

sustains the long-term maintenance of multi-lineage leukemia-initiating potential in CML stem cells. These findings underpin the paradoxical concept that FOXOs play opposite roles in the survival of CML stem cells vs. CML non-stem cells (Fig. 8.2).

The counterintuitive idea that FOXOs are active in leukemia stem cells can be extended to AML. In AML patients, Foxo signaling is activated in primitive $CD34^{+}lineage^{low}$ bone marrow cells (Sykes et al. 2011). Similarly, in a mouse AML model harboring the human *MLL-AF9* leukemic gene, mice transplanted with leukemia cells triply deficient for *Foxo1/3/4* display a longer latency to disease coupled with a decreased frequency of leukemia-initiating cells (leukemic-granulocyte-macrophage progenitor cells; L-GMP) compared to mice transplanted with leukemia cells possessing intact Foxo alleles (Sykes et al. 2011). Taken together, these results indicate that FOXOs are involved in the maintenance of leukemic stem cells in both CML and AML.

8.2.5 FOXO is Crucial for TKI Resistance in CML Stem Cells

The discovery of TKIs such as imatinib mesylate, nilotinib, and dasatinib that could directly inhibit the Abl kinase activity of the BCR–ABL oncoprotein was a dramatic breakthrough for the treatment of CML patients. However, although 95 % of imatinib-treated chronic phase CML patients survive for over 5 years (Druker et al. 2006), resistance to or intolerance of these drugs inevitably develops (Bhatia et al. 2003; Corbin et al. 2011; Cortes et al. 2004; Goldman and Gordon 2006; Savona and Talpaz 2008). Recent studies have demonstrated that TKIs are potent killers of differentiated leukemic cells, which are proliferating, but cannot deplete primitive CML stem cells, which are quiescent (Copland et al. 2006; Graham et al. 2002; Jorgensen et al. 2007; Konig et al. 2008a, b, c). It is possible that quiescent CML stem cells remaining after a first round of TKI treatment acquire mutations of the *BCR–ABL* gene itself. Such mutations would be manifested as TKI resistance in CML patients treated with a second round of TKI therapy (Gorre et al. 2001; Roumiantsev et al. 2002). Thus, many oncologists believe that TKI inhibitors coupled with novel therapeutics capable of eradicating quiescent CML stem cells may prevent disease recurrence and greatly benefit CML patients (Kavalerchik et al. 2008).

Because FOXOs play an essential role in the long-term maintenance of CML stem cells, we examined whether FOXOs also contribute to their TKI resistance. Although mice transplanted with *Foxo3a*$^{+/+}$ or *Foxo3a*$^{-/-}$ CML stem cells showed similar rates of lethality, the survival of mice that received *Foxo*-deficient CML stem cells and were treated with imatinib was significantly prolonged (Naka et al. 2010b). Furthermore, after imatinib administration, the number of residual CML stem cells present in mice transplanted with *Foxo3a*$^{-/-}$ CML stem cells was less than in mice transplanted with *Foxo3a*$^{+/+}$ CML stem cells. Interestingly, Muschen and colleagues have shown that the *BCL-6* proto-oncogene, which is a downstream effector of FOXO, is essential for both the maintenance of CML stem cells and the TKI resistance of ALL cells positive for the Philadelphia chromosome (Duy et al. 2011;

Hurtz et al. 2011). Therefore, therapeutics targeting FOXOs might help to eradicate CML stem cells in CML patients and render TKI-resistant cells in Ph⁺ALL patients susceptible to treatment.

8.2.6 *TGF-β Regulates FOXO Activity and the Self-Renewal Ability of CML Stem Cells*

Because TGF-β signaling regulates the Akt–FOXO pathway in normal HSCs, we investigated the impact of the TGF-β inhibitor Ly364947 on CML stem cell functions in vivo (Naka et al. 2010b). The administration of Ly364947 alone to CML-affected mice leads to Akt activation and decreased nuclear Foxo3a in CML stem cells, demonstrating that TGF-β is a critical regulator of Akt and Foxo in these cells in vivo. Notably, although administration of Ly364947 alone does not extend the survival of CML-affected mice, the administration of Ly364947 combined with imatinib treatment significantly reduces recipient lethality, CML stem cell frequency, and CML infiltration in the lung (Naka et al. 2010b). These findings indicate that the TGF-β-FOXO axis plays a critical role in the maintenance of TKI-resistant CML stem cells.

The sources of TGF-β that could support CML stem cell survival in vivo are under intense investigation. For normal HSCs, the activation of latent TGF-β is mediated by GFAP-expressing glial cells in the bone marrow (Yamazaki et al. 2011), making these cells important candidates for the source of TGF-β also maintaining the CML stem cell niche. In addition, Yokota et al. have demonstrated that osteoclasts are involved in the maintenance of dormant CML cells in the bone marrow through their release of TGF-β (Yokota et al. 2010). Recently, it was reported that an individual's genotype at the *killer immunoglobulin-like receptor* (*KIR*) genes is predictive of a complete cytogenetic response (CCyR) and survival rate in CML patients who are treated with imatinib (Marin et al. 2012). KIRs are expressed by natural killer (NK) cells, which are cells of the innate immune response. The physiological functions of NK cells, which include cytotoxicity and cytokine production, are controlled by a balance of inhibitory and activatory signals from cell surface receptors such as the KIRs. Significantly, NK cells are increased in number in CML patients treated with TKIs (Kreutzman et al. 2010; Yong et al. 2009). Marin et al. found that patients carrying the activatory receptor *KIR2DS1* have a significantly lower probability of achieving CCyR within 2 years of TKI treatment compared with patients who are *KIR2DS1*-negative (Marin et al. 2012). Importantly, NK cells expressing *KIR2DS1* secrete TGF-β (Ghio et al. 2009), implicating these cells as a possible source of the TGF-β driving the TKI resistance of CML stem cells.

Because TGF-β signaling is responsible for the maintenance of TKI-resistant CML stem cells in vivo, the suppression of TGF-β signaling so as to eradicate CML stem cells is a plausible therapeutic strategy. Although TGF-β-Foxo signaling is essential for the maintenance of both normal HSCs and CML stem cells, inhibition of TGF-β has a much more dramatic effect on CML stem cells than on normal HSCs. Notably, in the presence of imatinib, the treatment in vitro of

primitive $CD34^+$ human CML cells with TGF-β inhibitors attenuates their viability (Moller et al. 2007; Naka et al. 2010b). These results suggest that judicious inhibition of TGF-β-FOXO signaling may lead to efficient eradication of residual CML stem cells.

Section Summary: Because the activation of TGF-β signaling may be implicated in CML leukemogenesis, the application of TGF-β inhibitors to the treatment of CML patients is a promising therapeutic avenue. If detrimental effects on normal hematopoietic cells can be avoided, it is possible that the inhibition of TGF-β signaling might specifically deplete CML stem cells in patients with chronic phase CML and that a combination of a TGF-β inhibitor with TKIs might be effective in removing residual CML stem cells that survive therapy with TKI alone.

8.3 Conclusion

In this chapter, we have reviewed the multifaceted role of TGF-β signaling in leukemogenesis. As investigators have believed for many years, TGF-β signaling in normal HSCs helps to suppress the rise of several types of differentiated leukemia cells, including those present in AML, blast crisis CML, and pediatric ALL. Therefore, it is important to elucidate how the chromosomal translocations associated with these disorders generate oncoproteins that can inhibit TGF-β-mediated growth suppression. It is also important to recognize that TGF-β-FOXO signaling has the opposite effect on CML stem cells and is responsible for their survival and TKI resistance. Moreover, like normal HSCs, CML stem cells are quiescent, stress-resistant, and maintain their self-renewal ability within a niche. Thus, for the successful development of new small molecule drugs capable of eliminating CML stem cells, it may be advantageous to target the TGF-β signaling network (including elements of the Akt–FOXO pathway) and the niche cells that support it. The ultimate goal for this field is to develop novel therapeutics that can eradicate leukemia stem cells, thereby forestalling disease recurrence and providing concrete benefits for patients.

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